Lutropin increases phosphorylation of a 33 000-dalton ribosomal protein in rat tumour Leydig cells

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Addition of lutropin (luteinizing hormone, 'LH') and 3-isobutyl-1-methylxanthine to tumour Leydig cells stimulated phosphorylation of five proteins, of 17 000, 22 000, 24 000, 33 000 and 57 000 Da. Phosphorylation of these proteins coincided with increased pregnenolone production. Phosphorylation of a 33 000-Da protein was lutropin-dependent in Leydig cells isolated from a Leydig-cell tumour, from immature testes or from mature testes. In tumour Leydig cells this protein was present in the small ribosomal subunit. Incubation of tumour Leydig cells with either cycloheximide or puromycin inhibited both basal and lutropin-dependent pregnenolone production, by approx. 90% and 98% respectively. In contrast, basal pregnenolone production in Leydig cells from immature and mature testes was insensitive to cycloheximide or puromycin. Cycloheximide or puromycin increased phosphorylation of the 33 000-Da phosphoprotein by approx. 130% and 80% respectively (effect of lutropin/3-isobutyl-1-methylxanthine on phosphorylation: 100%). The molecular mass, the sub-cellular localization and the sensitivity to phosphorylation in the presence of inhibitors of protein synthesis indicate that the 33 000-Da protein could be similar to ribosomal protein S6.

The action of lutropin (luteinizing hormone, 'LH') on steroidogenesis in rat tumour Leydig cells is accompanied by activation of protein kinase and phosphorylation of proteins, and increased synthesis of specific proteins (Janszen *et al.*, 1978; Cooke *et al.*, 1979; Bakker *et al.*, 1981; for a review, see Rommerts & Brinkmann, 1981). It is not known whether, and if so how, phosphorylated proteins or newly synthesized proteins are related to the rate-limiting step in steroid production, i.e. the cholesterol-side-chain-cleavage activity in mitochondria. Lutropin-dependent phosphorylation of five proteins (17 000, 22 000, 24 000, 33 000 and 57 000 Da) has been demonstrated in rat tumour Leydig cells, and phosphorylation of these proteins coincided with increased steroid production (Bakker *et al.*, 1981). The 17 000-Da protein was isolated from the nuclear fraction, and the other four proteins were isolated from the postmitochondrial supernatant. None of the lutropin-dependent phosphoproteins was localized in mitochondria. Hence a direct effect of these phosphoproteins on the cholesterol-side-chain-cleavage enzyme appears unlikely.

Hormone [corticotropin (ACTH) or lutropin]-stimulated steroid production is dependent on protein synthesis (Schulster *et al.*, 1974; Cooke *et al.*, 1975; Farese *et al.*, 1980). Lutropin-dependent phosphoproteins present in the postmitochondrial supernatant may be involved in regulation of protein synthesis essential for steroidogenesis. In the present study we have therefore further investigated the localization and properties of the phosphoproteins present in the postmitochondrial supernatant, with particular reference to the ribosomes.

Materials and methods

Details of chemicals and methods used for isolation and incubation of tumour Leydig cells have been described previously (Bakker *et al.*, 1981). [³²P]P_i (carrier-free) and U-¹⁴C-labelled L-amino acids mixture (100–500 Ci/mol) were purchased from New England Nuclear, Boston, MA, U.S.A.

Isolation of testis Leydig cells from immature (21–23 days) and mature (90–100 days) Wistar rats (sub-strain R₁-Amsterdam) was performed as described by Janszen *et al.* (1976), except that no dextran-

centrifugation step was used. Incubation of cells was essentially as described by Bakker *et al.* (1981). In brief, cells were preincubated for 60 min and incubated with or without lutropin/3-isobutyl-1-methylxanthine as indicated (lutropin concentrations: 100 ng/ml for immature and mature testis Leydig cells, 1000 ng/ml for tumour Leydig cells; 3-isobutyl-1-methylxanthine: 0.25 mM). Incubations were performed in the presence of inhibitors of pregnenolone metabolism or [32 P]P_i, and with or without cycloheximide (89 μ M) or puromycin (120 μ M).

For isolation of ribosomes, tumour Leydig cells were incubated at 32°C under an atmosphere of 5% CO₂ in air in Krebs–Ringer buffer without phosphate (pH 7.3), with 0.2% glucose and 0.1% albumin (4×10^7 cells/2.5 ml). Labelling was performed for 60 min with 125 μ Ci of [32 P]P_i in the absence or

presence of lutropin/3-isobutyl-1-methylxanthine or cycloheximide, as indicated. Subsequently, cells were transferred to tubes containing 5 ml of 0.125 M-NaH₂PO₄/20 mM-NaF/82 mM-Tris (pH 7.3). Ribosomes were isolated as described by Walton & Gill (1973). Cells were centrifuged (10 min at 100 g), resuspended and homogenized in 0.25 M-sucrose/50 mM-Tris/HCl (pH 7.5)/25 mM-KCl/5 mM-MgCl₂/20 mM-NaF, with a Dounce glass homogenizer as described by Bakker *et al.* (1981). After removal of cell debris (10 min at 100 g) and nuclei plus mitochondria (10 min at 15 000 g), the isolated post-mitochondrial supernatant was adjusted to 4% Triton X-100 and 1% sodium deoxycholate. A ribosomal pellet was obtained by centrifugation in a Beckman SW 40 rotor for 2 h at 29 000 rev./min (105 000 g_{av.}). Ribosomes were resuspended in 150 μ l of TMD buffer [2 mM-Tris/

Fig. 1. Subcellular localization of the lutropin-dependent phosphoprotein of 33 000 Da and the effect of cycloheximide on its phosphorylation

Tumour Leydig cells were incubated for 60 min with [32 P]P_i in the absence (–) or presence (+) of lutropin/3-isobutyl-1-methylxanthine (LH/MIX): (a) without cycloheximide; (b) with cycloheximide. Ribosomes were isolated (see the Materials and methods section) and proteins were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Each panel shows the protein staining (on the left) and the corresponding radioautogram (on the right).

HCl (pH 7.5)/0.5 mM-MgCl₂/0.1 mM-dithiothreitol]. The suspension was adjusted to 250 mM-KCl by addition of 50 μ l of 1 M-KCl in TMD buffer, and layered on top of a linear 15–35% sucrose gradient (11 ml) in 10 mM-Tris/HCl (pH 7.5)/250 mM-KCl/2 mM-MgCl₂. The gradients were centrifuged in a Beckman SW 40 rotor for 15 h at 25 500 rev./min (80 900 g_{av}). The A_{260} pattern of the gradient was recorded. Fractions of the gradient containing the 40S and 60S subunits were pooled and adjusted to 10 mM-MgCl₂. Ribosomal proteins were precipitated by the addition of 0.7 vol. of ethanol. Finally, proteins were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Bakker *et al.*, 1981).

For determination of incorporation of ¹⁴C-labelled amino acids into proteins, cells were incubated in Minimal Essential Medium (Grand Island Biological Co., Grand Island, NY, U.S.A.), containing (per litre): 1.19 g of L-glutamine, 100 mg of streptomycin, 100 000 units of penicillin and 625 μ g of Fungizone. Incubations were performed at 32°C under air/CO₂ (19:1) in medium containing 1% foetal-calf serum. During 60 min of preincubation, tumour Leydig cells attached to Petri dishes (approx. 2×10^6 cells/dish). Incubations for 30 min in the presence of 10 μ Ci of ¹⁴C-labelled amino acids were performed in a final volume of approx. 850 μ l with or without lutropin/3-isobutyl-1-methylxanthine. After incubation, attached cells were washed with 5×1 ml of 0.9% NaCl and then 5×1 ml of 10% trichloroacetic acid (all at 4°C). Finally, cells were dissolved in 1 ml of 1 M-NaOH for determination of radioactivity and protein content. For determination of incorporation of [³²P]P_i into proteins, an incubation similar to that described above was performed with cells attached to Petri dishes (approx. 2×10^6 cells/dish). Incubations were for 60 min with 125 μ Ci of [³²P]P_i in 2 ml of Krebs–Ringer buffer without phosphate (pH 7.3), containing 0.2% glucose and 1% foetal-calf serum.

Results

Ribosomes were isolated from tumour Leydig cells that had been incubated for 60 min with [³²P]P_i with or without lutropin/3-isobutyl-1-methylxanthine. After homogenization of the cells, the 40S and 60S ribosomal subunits were separated on 15–35% sucrose gradients. Phosphorylated proteins obtained from the ribosomal subunits were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The lutropin-dependent 33 000-Da phosphoprotein was present in the 40S subunit (Fig. 1). Phosphorylation of a protein with the same molecular mass was stimulated by lutropin also in immature and mature testis Leydig cells (Fig. 2). Effects of inhibitors of protein synthesis on preg-

nenolone production and phosphorylation of proteins were investigated with concentrations of cycloheximide (89 μ M) and puromycin (120 μ M) as described by Cooke *et al.* (1975) for mature testis Leydig cells. These concentrations inhibited protein synthesis in all Leydig cells investigated to the same extent (approx. 95%). Cycloheximide or puromycin inhibited both basal and lutropin-dependent pregnenolone production in tumour Leydig cells by approx. 90% and 98% respectively (Table 1). In contrast, a specific phosphorylation of the 33 000-Da protein was detected in the presence of cycloheximide (Fig. 3) or puromycin (results not shown). Phosphorylation was increased by 130% and 80% in the presence of cycloheximide and puromycin respectively [stimulation by lutropin/3-isobutyl-1-methylxanthine = 100% (Table 1; see also Fig. 1)]. Moreover, a synergism of lutropin and cycloheximide was observed with regard to phosphorylation of the 33 000-Da protein (Table 1).

Fig. 2. Radioautogram showing the lutropin-dependent phosphorylation of the 33 000-Da protein in immature and mature testis Leydig cells

Leydig cells were incubated for 60 min with [³²P]P_i in the absence (–) or presence (+) of lutropin/3-isobutyl-1-methylxanthine (LH/MIX). Phosphorylated proteins were isolated and separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Arrows indicate lutropin-dependent phosphoproteins and their molecular masses in daltons.

Table 1. *Effect of inhibitors of protein synthesis on pregnenolone production and incorporation of [32 P] P_i into the 33 000-Da phosphoprotein in tumour Leydig cells*

Cells were incubated for 60 min with inhibitors of pregnenolone metabolism or [32 P] P_i as described previously (Bakker *et al.*, 1981). Parallel incubations (in duplicate) were performed in the absence (—) or presence (+) of cycloheximide (CX), puromycin (PUR) or lutropin/3-isobutyl-1-methylxanthine (LH/MIX). Peak heights of the 33 000-Da phosphoprotein measured from the densitograms were expressed as the percentage of the peak height of a standard protein of molecular mass 40 000 Da. Results are means $\pm$ s.d., for the numbers of different cell preparations shown in parentheses. Statistical analysis was done by Student's paired *t* test: n.s., not significant.

LH/MIX	Pregnenolone			Phosphate in 33 000-Da protein			Effect of inhibitor versus '+LH/MIX'
	CX (89 μ M)	PUR (120 μ M)	(ng/10 ⁶ cells)	Change versus '—LH/MIX'	(% of peak height)	Change versus '—LH/MIX'	
—	—	—	41.1 $\pm$ 9.1 (5)	—	69.3 $\pm$ 6.3 (4)	—	—
+	—	—	250.0 $\pm$ 31.1 (5)	<i>P</i> < 0.005	78.2 $\pm$ 3.7 (4)	<i>P</i> < 0.025	—
—	+	—	4.7 $\pm$ 1.6 (5)	<i>P</i> < 0.005	81.4 $\pm$ 7.1 (4)	<i>P</i> < 0.005	—
+	+	—	7.0 $\pm$ 1.7 (5)	<i>P</i> < 0.005	90.5 $\pm$ 4.3 (4)	<i>P</i> < 0.005	<i>P</i> < 0.005
—	—	+	4.2 $\pm$ 1.5 (5)	<i>P</i> < 0.005	76.3 $\pm$ 5.5 (3)	<i>P</i> < 0.025	—
+	—	+	5.1 $\pm$ 1.7 (5)	<i>P</i> < 0.005	82.3 $\pm$ 6.8 (3)	<i>P</i> < 0.01	n.s.

Fig. 3. *Effects of lutropin/3-isobutyl-1-methylxanthine and cycloheximide on phosphorylation of proteins of tumour Leydig cells*

Phosphorylated proteins were isolated from cells incubated in the presence of lutropin/3-isobutyl-1-methylxanthine with or without cycloheximide. Radioautograms obtained after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis were scanned with a Gilford spectrophotometer (for full details see Bakker *et al.*, 1981). Arrows indicate lutropin-dependent phosphoproteins. The large arrow indicates the additional phosphorylation of the 33 000-Da phosphoprotein observed in the presence of cycloheximide. Numerical values are molecular masses in daltons.

Kinetic studies of the inhibition of pregnenolone production and the phosphorylation of the 33 000-Da protein were performed with cycloheximide. The decrease with time in basal and lutropin-stimulated

pregnenolone production after addition of cycloheximide was more or less the same, which indicated the possible involvement of a protein(s) with a half-life of approx. 6 min (Fig. 4). A gradually

Fig. 4. Kinetics of the effect of cycloheximide on pregnenolone production by tumour Leydig cells. Cells were incubated for 30 min with or without lutropin/3-isobutyl-1-methylxanthine (LH/MIX). Subsequently, cycloheximide was added and pregnenolone production was determined during 5 min periods. Results shown are the mean values obtained in three experiments with duplicate incubations. Further data for the regression lines shown for the incubations without and with lutropin/3-isobutyl-1-methylxanthine respectively: number of mean values, 11 and 12; correlation coefficient, -0.85 and -0.96 ; half-time of the decrease, 5.8 min and 6.0 min.

increasing phosphorylation of the 33 000-Da protein was observed during incubations for 60 min in the presence of cycloheximide and with or without lutropin/3-isobutyl-1-methylxanthine (results not shown). Unlike tumour Leydig cells, basal pregnenolone production in immature and mature testis Leydig cells was not inhibited by either cycloheximide (Table 2) or puromycin (results not shown).

Increased phosphorylation of the 33 000-Da ribosomal protein might be the consequence of increased specific radioactivity of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ caused by increased uptake of $^{32}\text{P}\text{P}_i$ in the presence of lutropin/3-isobutyl-1-methylxanthine. On the other hand, phosphorylation of the 33 000-Da protein may be involved in regulation of protein synthesis. Therefore incorporation of $^{32}\text{P}\text{P}_i$ and ^{14}C -labelled amino acids into proteins was determined. However, no effects of lutropin/3-isobutyl-1-methylxanthine could be demonstrated (Table 3).

Table 2. Pregnenolone production in Leydig cells isolated from immature testes, mature testes and a Leydig-cell tumour. Leydig cells were incubated for 60 min in the absence (—) or presence (+) of lutropin/3-isobutyl-1-methylxanthine (LH/MIX) or cycloheximide (CX). Results are means $\pm$ s.d. for the numbers of different cell preparations shown in parentheses. Statistical analysis was done by Student's paired *t* test: n.s., not significant.

LH/MIX	CX (89 μM)	Immature Leydig cells			Mature Leydig cells			Tumour Leydig cells		
		(ng/10 ⁶ cells)	Effect of LH/MIX	Effect of CX	(ng/10 ⁶ cells)	Effect of LH/MIX	Effect of CX	(ng/10 ⁶ cells)	Effect of LH/MIX	Effect of CX
—	—	0.5 $\pm$ 0.1 (3)	—	—	7.2 $\pm$ 1.1 (2)	—	—	41.1 $\pm$ 9.1 (5)	—	—
+	—	17.8 $\pm$ 4.3 (3)	$P < 0.025$	—	39.5 $\pm$ 3.5 (2)	$P < 0.025$	—	250.0 $\pm$ 31.1 (5)	$P < 0.005$	—
—	+	0.5 $\pm$ 0.1 (3)	—	n.s.	6.6 $\pm$ 1.5 (2)	—	n.s.	4.7 $\pm$ 1.6 (5)	—	$P < 0.005$
+	+	0.7 $\pm$ 0.1 (3)	$P < 0.05$	$P < 0.025$	6.6 $\pm$ 0.6 (2)	n.s.	$P < 0.025$	7.0 $\pm$ 1.7 (5)	$P < 0.005$	$P < 0.005$

Table 3. Incorporation of [32 P] P_i and 14 C-labelled amino acids into proteins of tumour Leydig cells

Cells were incubated in the absence (–) or presence (+) of lutropin/3-isobutyl-1-methylxanthine with either 125 μ Ci of [32 P] P_i or 10 μ Ci of 14 C-labelled amino acids. Incorporation of label was determined as described in the Materials and methods section. Results shown are values obtained with duplicate incubations (mean $\pm$ range).

Lutropin/3-isobutyl-1-methylxanthine	Expt. 1 ($10^{-3} \times ^{32}\text{P c.p.m./}$ 60 min per mg)	Expt. 2 ($10^{-3} \times ^{14}\text{C d.p.m./}$ 30 min per mg)	Expt. 3 ($10^{-3} \times ^{14}\text{C d.p.m./}$ 30 min per mg)
–	587.0 $\pm$ 57.1	101.4 $\pm$ 5.4	165.1 $\pm$ 3.5
+	523.1 $\pm$ 34.2	101.8 $\pm$ 10.8	162.4 $\pm$ 6.5

Discussion

The aim of the present study was to investigate further the localization of lutropin-dependent phosphoproteins demonstrated in tumour Leydig cells (Bakker *et al.*, 1981), which might be involved in regulation of steroidogenesis. The results indicate that a lutropin-dependent phosphoprotein of 33 000 Da was present in the 40S ribosomal subunit isolated from tumour Leydig cells. Cycloheximide and puromycin also stimulated phosphorylation of this protein, under conditions in which basal and lutropin-dependent pregnenolone productions were inhibited. The decrease with time in pregnenolone production under basal and stimulated conditions was about the same, which suggests the possible involvement of a protein(s) with a short half-life (approx. 6 min).

Unlike tumour Leydig cells, basal pregnenolone production in immature and mature testis Leydig cells was not inhibited by cycloheximide (or puromycin). Basal pregnenolone production expressed as ng/60 min per 10^6 cells in tumour Leydig cells is relatively high (approx. 80 times and 6–7 times the basal pregnenolone production in immature and mature testis Leydig cells respectively). Apart from different contents of Leydig cells in the various preparations (cf. Janszen *et al.*, 1976; Cooke *et al.*, 1979), the higher basal pregnenolone production in tumour Leydig cells may be due to increased basal amounts of cyclic AMP (cf. Cooke *et al.*, 1976, 1979). This would explain the comparable kinetics of inhibition by cycloheximide of pregnenolone production under basal and stimulated conditions (Fig. 4).

Effects of protein-synthesis inhibitors on phosphorylation of ribosomal protein S6 have been reported (Gressner & Wool, 1974; Lastick & McConkey, 1980). The molecular mass, the subcellular localization and the sensitivity to phosphorylation in the presence of inhibitors of protein synthesis suggest that the 33 000-Da protein could be similar to protein S6. Immature and mature testis Leydig cells also showed a lutropin-dependent phosphoprotein of 33 000-Da, which suggests that in Leydig cells from rat testis lutropin-dependent phosphorylation of ribosomal protein S6 occurs.

The increased phosphorylation of ribosomal protein S6 in the presence of inhibitors of protein synthesis has been explained by inhibition of metabolism of cyclic nucleotides (Gressner & Wool, 1974; Leader, 1980). However, increased amounts of cyclic AMP will probably stimulate cyclic AMP-dependent protein kinase in a non-specific way, resulting in increased phosphorylation of all cyclic AMP-dependent phosphoproteins. In the present study we observed an effect only on phosphorylation of protein S6. Therefore, increased phosphorylation of a specific protein in the presence of cycloheximide or puromycin cannot be easily explained by a non-specific increase in cyclic AMP. Moreover, since no effect of lutropin/3-isobutyl-1-methylxanthine could be detected on the incorporation of [32 P] P_i into proteins phosphorylation owing to changes in the specific radioactivity of [γ - 32 P]ATP is unlikely. The increase in phosphorylation of ribosomal protein S6 was higher in the presence of cycloheximide than of puromycin, which may be related to the different actions of cycloheximide and puromycin. The action of cycloheximide (inhibition of the peptidyl-translocation step of elongation) involves binding to the ribosome, resulting in inhibition of passage of the ribosome along the mRNA. The action of puromycin, however, involves binding to the growing peptide chain, which causes release of this peptide chain and dissociation of the ribosomal subunits. Hence, the greater stimulation of phosphorylation of ribosomal protein S6 in the presence of cycloheximide shown in our study and in other studies (Gressner & Wool, 1974; Lastick & McConkey, 1980) might be due to steric hindrance at the ribosome. Phosphorylation of protein S6 in the presence of cycloheximide increased with time, which may indicate that its phosphorylation continues under the influence of the basal activity of probably cyclic AMP-dependent protein kinase (see above), whereas dephosphorylation is impaired or even impossible, owing to the presence of cycloheximide. During incubations of ribosomes isolated from bovine adrenal (Walton & Gill, 1973) and corpus luteum (Azhar & Menon, 1975) with cyclic AMP-dependent protein kinase, phosphorylation of a single protein in the 40S

subunit and of six to twelve proteins in the 60S subunit has been observed. The greater number of phosphoproteins in those studies may represent an artifact *in vitro* (see, e.g., Krebs & Beavo, 1979).

Phosphorylation of ribosomal protein S6 has been frequently related to increased protein synthesis (Leader, 1980). However, in tumour Leydig cells incorporation of amino acids into proteins was not stimulated by lutropin/3-isobutyl-1-methyl-xanthine during 30 min of incubation, whereas lutropin-dependent phosphorylation of protein S6 was almost maximal after 5 min of incubation (Bakker *et al.*, 1981). It is possible, however, that synthesis of some specific protein, i.e. the rapidly-turning-over protein(s) required for hormone-dependent steroid production, is increased via phosphorylation of proteins involved in protein synthesis.

It is known that cyclic AMP can stimulate phosphorylation of protein S6 in some other cell types: hepatocytes (Gressner & Wool, 1976), thymocytes (Wettenhall & Howlett, 1979), HeLa cells (Lastick & McConkey, 1980). Moreover, cyclic AMP can stimulate synthesis of specific proteins such as tyrosine aminotransferase (Wicks *et al.*, 1969; Snoek *et al.*, 1981) and phosphoenolpyruvate carboxykinase (Iynedjian & Hanson, 1977) in rat hepatocytes and hepatoma cells, with no detectable increase in general protein synthesis. The increased synthesis of tyrosine aminotransferase in rat hepatoma cells in response to cyclic AMP appears to reflect an increase in the rate of initiation on the tyrosine aminotransferase mRNA (Snoek *et al.*, 1981). In Leydig cells a similar mechanism could operate for the induction of the rapidly-turning-over protein(s) required for lutropin-dependent steroid production. The lutropin-dependent phosphorylation of ribosomal protein S6 and proteins of 22000 and 24000 Da (Bakker *et al.*, 1981) might participate in this mechanism of specific translation.

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